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Cultivation of *Podospora anserina* on soybean hulls results in an efficient enzyme cocktail for plant biomass hydrolysis

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Abstract

The coprophilic ascomycete fungus *Podospora anserina* was cultivated on three different plant biomasses, i.e. cotton seed hulls (CSH), soybean hulls (SBH) and acid-pretreated wheat straw (WS) for four days, and the potential of the produced enzyme mixtures was compared in the enzymatic saccharification of the corresponding lignocellulose feedstocks. The enzyme cocktail *P. anserina* produced after three days of growth on SBH showed superior capacity to release reducing sugars from all tested plant biomass feedstocks compared to the enzyme mixtures from CSH and WS cultures. Detailed proteomics analysis of the culture supernatants revealed that SBH contained the most diverse set of enzymes targeted on plant cell wall polymers and was particularly abundant in xylan, mannan and pectin acting enzymes. The importance of lytic polysaccharide monooxygenases (LPMOs) in plant biomass deconstruction was supported by identification of 20 out of 33 AA9 LPMOs in the SBH cultures. The results highlight the suitability of *P. anserina* as a source of plant cell wall degrading enzymes for biotechnological applications and the importance of selecting the most optimal substrate for the production of enzyme mixtures.

Keywords

Proteomics; saccharification; plant biomass; *Podospora anserina*; carbohydrate active enzymes; auxiliary activities

Highlights

- *P. anserina* produced an efficient enzyme set for saccharification in SBH cultures
- The most diverse set of CAZymes was detected in SBH secretomes
- The enzyme cocktail from SHB was superior in hydrolysis of different plant biomasses
- The secretome from SBH was rich in xylan, mannan and pectin acting enzymes

Abbreviations

ABF, α -arabinofuranosidase; ABX, exoarabinanase; AE, acetyl esterase; AGD, α -glucosidase; AGL, α -1,4-D-galactosidase; AGU, α -glucuronidase; AMY, amylase; APO, aromatic peroxygenase; AXH, arabinoxylan arabinofuranohydrolase; BGL, β -1,4-glucosidase; BXL, β -xylosidase; CAZy, carbohydrate active enzyme; CBH, cellobiohydrolase; CDH, cellobiose dehydrogenase; CMCase, carboxymethyl cellulase; CSH, cotton seed hull; DNS, dinitrosalicylic acid; EGL, endoglucanase; FAE, feruloyl esterase; FDR, false discovery rate; GAL, endogalactanase; GE, glucuronoyl esterase; GMC, glucose-methanol-choline oxidoreductase; GLA, glucoamylase; LAC, β -1,4-D-galactosidase; LPMO, lytic polysaccharide monooxygenase; MEA, malt extract agar; MM, minimal medium; MAN, endo- β -1,4-mannanase; MND, β -1,4-mannosidase; MCO, multicopper oxidase; PCA, principal component analysis; PL, pectin lyase; PME, pectin methyl esterase; *p*PN, *p*-nitrophenol; PSM, peptide spectrum match; RGAE, rhamnogalacturonan acetyl esterase; RHA, α -rhamnosidase; SBH, soybean hulls; WS, wheat straw; XLN, endoxylanase.

1 Introduction

Podospora anserina is a dung inhabiting ascomycete fungus that has traditionally been studied as a model species for several biological questions, such as ageing, prion mechanisms and sexual development [1–4]. The genome sequence of *P. anserina* revealed that this species possesses a large and highly specialized gene repertoire encoding hydrolytic and oxidative carbohydrate active enzymes (CAZymes; [5]) that match with the carbon sources present in the natural biotope of the fungus [6]. Therefore, more recently the characterization of the lignocellulose acting enzyme machinery of *P. anserina* has gained increasing attention and led e.g. to recombinant production and biochemical studies of several of its CAZymes [7].

As a late coloniser of herbivorous dung [8], *P. anserina* has adapted to grow on lignocellulosic substrates from which easily degradable carbon sources have already been largely consumed by primary colonising species. This is reflected in its genome content comprising a large repertoire of CAZymes putatively targeted to (crystalline) cellulose degradation and including both hydrolytic and oxidative activities. These include e.g. several glycoside hydrolases from CAZy families GH6, GH7, GH45 and GH131, and, even more noticeably, 33 putative lytic polysaccharide monooxygenases (LPMOs) from family AA9 [6]. In addition, *P. anserina* possesses an abundant xylan degrading machinery with several candidate enzymes from GH10, GH11, GH3 and GH43 families. Also, the relatively high number of putative esterases possibly indicates the ability of *P. anserina* to detach xylan from lignin and utilise this more recalcitrant fraction of lignocellulose as a carbon source. Moreover, compared to other plant biomass degrading ascomycete species, the *P. anserina* genome encodes a large number of oxidative auxiliary activities connected to lignin modification, including several genes for putative AA1_3 laccase-like multicopper oxidases (MCOs) and AA3_2 H₂O₂-producing enzymes. It also possesses three cellobiose dehydrogenases (CDHs, AA1_3 and AA8), which may function in the degradation of all main lignocellulose polymers [9,10].

The ability of *P. anserina* to degrade recalcitrant components of plant biomass makes it a highly interesting species as a source of novel enzymes for plant biomass pre-treatment in biotechnological applications. To further explore this ability, we cultivated *P. anserina* on three lignocellulosic biomass substrates: cotton seed hulls (CSH), soybean hulls (SBH) and pre-treated wheat straw (WS). The choice for CSH and SBH was based on their potential as a biorefinery feedstock as they are abundant agricultural waste streams. In addition, we included acid-pre-treated WS, as it is a currently used feedstock in biorefineries and therefore presents a real case to test the potential of this fungus. We used a combination of enzyme and

saccharification assays with proteomics to obtain an in depth insight into the abilities of *P. anserina*.

2 Materials and methods

2.1 Fungal cultivations

Podospora anserina S mat⁺ was kindly provided by Dr. Fabienne Malagnac (Institut de Génétique et Microbiologie, UMR 8621, Université Paris Sud-CNRS, France). The strain was maintained on 2% (w/v) malt extract agar (MEA) plates at 4°C. For precultures, medium that contained 1% of fructose and *P. anserina* modified minimal medium (MM, 0.25 g/l KH₂PO₄; 0.3 g/l K₂HPO₄; 0.25 g/l MgSO₄·7H₂O; 0.05 mg/l thiamine 0.25 µg/l biotin; 25 µg/l boric acid; 2.5 mg/l ZnSO₄; CuSO₄ 0.5 mg/l; MnSO₄ 125 µg/l and vishniac solution [11] at pH 7.0) was prepared. The inoculum was obtained by mixing a quarter of mycelium-covered 2% MEA agar plate with 100 ml of MM, and 20 ml of this mixture was used to inoculate 400 ml MM medium supplemented with 1% fructose. After 3 days of shaken (200 rpm) incubation at 27°C, the fungal mycelium was collected by filtration and washed with MM. 2 g wet biomass was used to inoculate 100 ml MM supplemented with 3% of powdered cotton seed hulls (CSH), soybean hulls (SBH) and wheat straw (WS) as carbon source. WS treated with 20% HCl for 24 h and then washed and dried, was provided by Green Sugar GmbH, Germany. Biological duplicate cultures were incubated for 4 days at 27°C and shaken (200 rpm).

Culture supernatants from each cultivation were collected from day 1 to day 4 for further analyses.

2.2 Enzyme activity assays

Cell free samples were used for measurement of extracellular CAZyme activities. All measurements were performed as three technical replicates, and the averages and standard deviations were calculated using two biological replicate cultures and three technical replicate measurements.

α -Arabinofuranosidase (ABF), cellobiohydrolase (CBH), α -1,4-D-galactosidase (AGL), β -1,4-D-galactosidase (LAC), glucoamylase (GLA), β -1,4-glucosidase (BGL), β -1,4-mannosidase (MND) and α -rhamnosidase (RHA) activities were determined by using *p*-nitrophenol (*p*PN) -linked substrates (Sigma-Aldrich) as described previously [12]. Reaction mixtures were incubated at 30°C for 4 h after which the reactions were terminated by adding 100 μ l 0.5 M sodium carbonate. The amount of the released *p*NP was measured spectrophotometrically (FLUOstar OPTIMA, BMG Labtech) at 405 nm. Activities are expressed as nmol *p*NP/ml of sample/min.

Endoxylanase (XLN) and carboxymethyl cellulase (CMCase) activities were determined using beechwood xylan (Sigma-Aldrich) and carboxymethyl cellulose (CMC, Sigma-Aldrich) as substrates, respectively. Reaction mixtures containing 5 μ l of culture supernatant and 45 μ l of 1% substrate in 50 mM sodium acetate, pH 5.0, were incubated at 50°C for 30 min. Dinitrosalicylic acid (DNS) assay was used to measure the amount of released sugars [13]. Reaction was stopped by 75 μ l DNS solution and boiling for 30 min. The absorbance was measured at 550 nm. Activities are expressed as nmol of glucose released/ml of sample/min.

2.2 SDS-PAGE and protein concentration measurement

For protein profiling, 30 μ L of culture supernatant from each sample was loaded on 12% SDS-PAGE gel and stained by silver staining. SigmaMarker wide range (Sigma-Aldrich) was used as a molecular mass marker.

The protein content of the culture supernatants was estimated by using BioRad protein kit (BioRad) following the instructions of the manufacturer. Bovine serum albumin (Sigma) was used as standard and the results are expressed in mg/l.

2.3 Composition analysis of the used substrates

Neutral carbohydrate composition of CSH, SBH and WS was analysed according to Englyst and Cummings, 1984 [14], using inositol as an internal standard. Samples were treated with 72% (w/w) H₂SO₄ (1 h, 30°C) followed by hydrolysis with 1 M H₂SO₄ for 3 h at 100°C and the constituent sugars released were derivatised and analysed as their alditol acetates using gas chromatography.

2.4 Saccharification

Culture supernatants obtained from the CSH, SBH and WS cultures of *P. anserina* after three days of cultivation were used for saccharification of CSH, SBH and acid-pre-treated WS. The 500 µl reactions were conducted with 3% (w/v) solid substrate in 50 mM Na-phosphate buffer, pH 5.0, in the presence of 0.02% sodium azide. Total protein loading in each reaction was 621 µg. The reactions were incubated for 24 h, 300 rpm, at 50°C, after which the amount of reducing sugars was determined using DNS method. The experiments were performed in technical triplicates.

2.5 Proteomics analysis

2.5.1 Protein separation and digestion

Samples were ran on a 12% Bis-Tris 1D SDS-PAGE gel (BioRad) for 2-3 cm and stained with colloidal coomassie dye G-250 (Gel Code Blue Stain Reagent, Thermo Scientific). The lane was cut into three bands, which were treated with 6.5 mM dithiothreitol

(DTT) for 1 h at 60°C for reduction and 54 mM iodoacetamide for 30 min for alkylation. The proteins were digested overnight with trypsin (Promega) at 37°C. The peptides were extracted with 100% acetonitrile and dried in a vacuum concentrator.

2.5.2 Mass spectrometry: RP-nanoLC-MS/MS

Samples were resuspended in 10% formic acid / 5% DMSO and 30% of the sample was analyzed using a Proxeon Easy-nLC100 (Thermo Scientific) connected to an Orbitrap Q-Exactive mass spectrometer. Samples were first trapped (Dr Maisch Reprosil C18, 3 µm, 2 cm x 100 µm) before being separated on an analytical column (Agilent Poroshell EC-C18, 2.7 µm, 40 cm x 50 µm), using a gradient of 60 min at a column flow of 150 nl/min. Trapping was performed at 8 µL/min for 10 min in solvent A (0.1 M acetic acid in water) and the gradient was as follows 7- 30% solvent B (0.1 M acetic acid in acetonitrile) in 31 min, 30-100% in 3 min, 100% solvent B for 5 min, and 7% solvent B for 13 min. Nanospray was performed at 1.7 kV using a fused silica capillary that was pulled in-house and coated with gold (o.d. 360 µm; i.d. 20 µm; tip i.d. 10 µm). The mass spectrometers were used in a data-dependent mode, which automatically switched between MS and MS/MS. Full scan MS spectra from m/z 350 – 1500 were acquired at a resolution of 35,000 at m/z 400 after the accumulation to a target value of 3E6. Up to ten most intense precursor ions were selected for fragmentation. HCD fragmentation was performed at normalised collision energy of 25% after the accumulation to a target value of 5e4. MS2 was acquired at a resolution of 17,500 and dynamic exclusion was enabled (exclusion size list 500, exclusion duration 10 s).

2.5.3 Data analysis

Raw files were processed using Proteome Discoverer 1.4 (version 1.4.1.14, Thermo Scientific, Bremen, Germany). Database search was performed against the *P. anserina*

database (JGI Mycocosm, <http://genome.jgi.doe.gov/Podan2/Podan2.home.html>) using Mascot (version 2.5, Matrix Science, UK) as the search engine. Carbamidomethylation of cysteines was set as a fixed modification and oxidation of methionine was set as a variable modification. Trypsin was specified as enzyme and up to two miss cleavages were allowed. Data filtering was performed using percolator [15], resulting in 1% false discovery rate (FDR). Additional filters were; search engine rank 1 peptides and ion score >20. Raw files corresponding to one sample were merged into one result file. The MS proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [16] partner repository with the dataset identifier PXD005170 and 10.6019/PXD005170. The number of peptide spectrum matches (PSMs) for each protein in each sample were merged into one matrix file. Principal component analysis (PCA) was performed on the resulting matrix table with the FactoMineR package, a package written in R dedicated to multivariate exploration data analysis [17].

3 Results

3.1 Extracellular enzyme activities and protein profiles in *P. anserina* cultures

Enzyme activities of selected hydrolytic CAZymes were determined from cultures where powdered CSH, SBH or WS were used as sole carbon sources during 4 days of cultivation (Fig. 1). The highest activities were detected from the cultures with SBH as a carbon source, which most probably was due to the higher protein production produced on this substrate (Supplementary Table 1). While CBH, BGL and XLN activities were detected in all studied media, LAC, ABF, AGL and CMCase activities were only observed in SBH supernatants (Fig. 1B). The highest CBH activity was determined at the third day of growth in all three cultures. Activities measured in CSH and WS supplemented media were at similar level (2.1 and 1.5 nmol of *p*NP released/min/ml, respectively; Fig. 1A, C), while in SBH

medium, 6- and 9-fold higher CBH activity was detected (Fig. 1B). The highest BGL (3.5 nmol of *p*NP released/min/ml) and XLN (4.1 nmol of glucose released/min/ml) activities were also detected from the SBH cultures at the fourth and third day of growth, respectively (Fig. 1B). Compared to CSH and WS cultures, 3- and 5-fold higher BGL activity, and 8- and 3-fold higher XLN activity was produced in the SBH cultures of *P. anserina* (Fig. 1B). No MND, GLA and RHA activity were detected in the culture supernatants.

[Figure 1]

SDS-PAGE analysis revealed that *P. anserina* produced variable sets of extracellular proteins over time in different plant biomass containing cultures (Supplementary Fig. 1), thus indicating adaptation to each carbon source. In line with the detected enzyme activities, higher amount of extracellular proteins was produced in SBH cultures compared to CSH and WS cultures (Supplementary Table 1). As expected, the protein amount increased in the cultures during the cultivation resulting in 171, 376 and 173 mg/l in CSH, SBH and WS, respectively, at the third day (Supplementary Table 1E), which was selected for saccharification experiments (see 3.3).

3.2 Carbohydrate composition of the plant biomass substrates

The plant biomass substrates used in this work consisted of one monocot, i.e. WS, and two dicots, i.e. CSH and SBH, which substantially differ in their chemical composition (Supplementary Table 2). The glucose content of CSH is approximately 10 mol% lower compared to SBH and WS. In contrast, the xylan content of CSH is significantly higher than that of the two other substrates. Furthermore, SBH contains mannan and distinguishes from

the others with its high pectin content shown by the presence of galactose, arabinose and uronic acids (Supplementary Table 2).

3.3 Saccharification of lignocellulosic substrates by the enzyme mixtures produced by *P. anserina*

Since the highest enzyme activities were detected after 3 days of *P. anserina* growth on CSH, SBH and WS cultures, these extracellular enzyme mixtures were selected for saccharification assays of the lignocellulosic feedstocks by measuring the amount of released reducing sugars (Fig. 2). The enzyme cocktail originating from SBH cultures hydrolysed all tested plant biomass substrates resulting in release of 0.7, 1.2 and 0.3 g/l reducing sugars from CSH, SBH and acid-pre-treated WS, respectively. In contrast, the enzyme set from CSH was able to saccharify only SBH, whereas enzymes from WS hydrolysed CSH and SBH. Moreover, the superiority of the enzyme mixture produced on SBH was seen in that it released more than 2-fold higher amount of reducing sugars from CSH compared to enzymes produced on WS, and 4- and 2-fold higher amount from SBH compared to enzymes produced on CSH and WS, respectively (Fig. 2). In line with the chemical composition of the biomasses, acid-pre-treated WS was the most recalcitrant substrate lacking the easily hydrolysable sugars compared to the non-pre-treated WS (Supplementary Table 2), and only the enzyme mixture from SBH cultures accomplished to release reducing sugars from it. Surprisingly, in the studied conditions, the enzymes from CSH and WS cultures did not hydrolyse the substrates on which they were produced.

[Figure 2]

3.4 Proteomics of *P. anserina* cultures

In order to study *P. anserina* enzyme mixtures more thoroughly, the culture liquids from the first and third day cultures were selected for proteomics analysis, based on the detected protein profiles and enzyme activities. The PCA analysis showed that the duplicate samples were very similar to each other (Supplementary Fig. 2). *P. anserina* produced the highest number of selected plant cell wall degradation related enzymes on SBH at both studied time points. Their number was slightly higher at the early time point of SBH (114) compared to WS (99) and substantially higher compared to CSH with 47 detected proteins (Fig. 3, Supplementary Table 1). Of these proteins, 39 (83%), 106 (93%) and 88 (89%) from CSH, SBH and WS, respectively, have predicted secretion signal peptides (Supplementary Table 1C). While the number of the plant cell wall degradation related enzymes stayed almost the same after 3-day cultivation in SBH (116) and WS (98), it notably increased in CSH (103). Putative secretion signals were present in 89 (86%), 104 (90%) and 89 (91%) of the selected enzymes detected from CSH, SBH and WS cultures, respectively. The actual number of secreted proteins may be higher than is suggested by the *P. anserina* genome annotation, since half of the genes without a secretion signal sequence that were selected for the analysis (Supplementary Table 1C) were revealed to contain a putative secretion signal after manual correction of the gene model (Supplementary Table 1F).

The number of enzymes that were unique to SBH was high (22) compared to CSH (1) and WS (11) after 1-day cultivation. The number of enzymes common to all three cultures increased from 40 to 69, while the number of enzymes shared between SBH and WS notably decreased (from 47 to 14) during the cultivation (Fig. 3). Significant increase in the number of unique proteins in CSH cultures (from 1 to 17) was detected after 3 days of cultivation, while it did not change in SBH (22) and slightly diminished in WS (from 11 to 9).

[Figure 3]

With respect to cellulose degradation, several CBH, endoglucanase (EGL) and BGL enzymes were produced by *P. anserina* (Supplementary Table 1). GH6 and GH7 CBHs were highly produced in WS cultures at both time points. Furthermore, one GH6 and one GH1 BGL were among the 15 most highly produced, and one GH7 CBH was the most abundant CAZyme in WS supernatants at both time points (Supplementary Table 1C). GH5 EGLs were present at the highest level in SBH cultures. In accordance with the highest release of reducing sugars from all plant biomass substrates used in this work, the highest CAZyme activities were detected from the SBH cultures of *P. anserina*. However, the measured enzyme activities did not correlate in all cases with the proteomics analysis. For example, CMCase activity was measured only from the supernatants obtained from SBH. This was most probably due to inactivation or instability of the enzymes in the other samples. In addition, it is possible that not all enzymes produced by *P. anserina* are active on the *p*NP-linked substrates. No notable differences between the cultures were detected in the abundance of BGLs.

Proteomics analysis revealed that majority (20 out of 33) of the AA9 LPMOs were present in *P. anserina* SBH cultures (Supplementary Table 1). Most of these (18) were produced after 3 days of growth, while 15 were identified at the early time point. Also, a large variety of LPMOs were detected in WS cultures with 15 isoenzymes present in both time points, and two of them were among the 15 most highly detected CAZymes (Supplementary Table 1C). A narrower selection of 6 LPMOs was produced in CSH medium after 1 day of cultivation, but 15 representatives were recognised on day 3. In addition, LPMOs were generally produced in higher amounts in the later time point of SBH than of CSH and WS containing cultures. The number of unique LPMOs was also highest in SBH

with 3 and 4 representatives in day 1 and 3, respectively (Fig. 3). In line with the high production of LPMOs, all three *P. anserina* putative AA8-AA3_1 CDHs were also detected in the studied cultures. One CDH, Pa_7_2650, was among the 15 most abundantly produced plant cell wall acting proteins at both time points of all cultures, and another CDH, Pa_0_280 also in the 3-day CSH and WS supernatants (Supplementary Table 1C).

The ample ability of *P. anserina* to degrade hemicelluloses was seen when the proportions of the selected plant cell wall acting enzymes were compared with their putative substrates (Fig. 4). Accordingly, galactomannan degrading GH5 and GH26 endo- β -1,4-mannanases (MANs) and GH27 AGLs were abundant in SBH cultures compared to CSH and WS cultures. In addition, one GH26 MAN, one GH5_5 MAN and one GH2 MND belonged to the 15 CAZymes that were most highly produced in SBH cultures (Supplementary Table 1C). However, AGL activity was only detected from 4-day culture liquids from SBH. Especially, GH5 endo- β -1,4-mannanases were almost exclusively detected from SBH supernatants, resulting also with GH5 proteins detected uniquely from SBH (Fig. 3). This is in accordance with the chemical composition of SBH containing 7.1 and 4.0 mol% of mannose and galactose, respectively (Supplementary Table 2, [18]). GH2 mannosidases were notably produced at the later time point in SBH and CSH, although no MND activity was detected from any of the studied culture supernatants.

[Figure 4]

Xylan degrading activities were also highly present in *P. anserina* secretomes (Fig. 4). XLNs from GH10 were highly produced after 3 days of growth in CSH and SBH media, while those from GH11 were abundant in WS and SBH (Supplementary Table 1). GH11 XLN (Pa_1_1010) was the most abundant plant cell wall degrading enzyme in SBH in both

time points (Supplementary Table 1C). From putative β -xylosidases (BXLs), the enzymes classified to GH43 were slightly more abundant in SBH than in the two other substrates. GH62 arabinoxylan arabinofuranohydrolase (AXH) was highest on SBH, and GH115 and GH67 α -glucuronidases (AGUs) on CSH. The high xylan degrading potential of the 3-day SBH supernatant used for the saccharification was also evidenced by a GH10 XLN, GH62 AXH and GH43 BXL that were among the 15 most highly detected proteins. From putative xyloglucan depolymerising enzymes, GH12 Pa_4_6020 was most abundant in WS, whereas GH74 Pa_4_7820 was highest in SBH. The same was true for two CE15 glucuronoyl esterases (GEs, Pa_0_910 and Pa_5_11620) and a putative CE16 acetyl esterase (AE, Pa_5_11290), which were more abundant in the *P. anserina* proteome from 3-day SBH cultivations compared to CSH and WS. In addition, CE15 Pa_5_11620 was unique for SBH secretomes (Fig. 3). However, putative CE1 feruloyl (FAEs) and AEs were detected in all samples. GH2 LACs, and GH43 and GH51 ABFs, which may act on several polysaccharides, were almost exclusively produced in SBH. This is in line with the detection of LAC and ABF activity only from SBH cultures. Interestingly, highly variable GH43 isoenzymes were produced on different substrates on day 3 (Fig. 3).

Pectin acting enzymes were prevalent on SBH, while they were absent or lowly produced on CSH and WS (Fig. 4, Supplementary Table 1). These included CE12 rhamnogalacturonan acetyl esterase (RGAE, Pa_1_22170), CE8 pectin methyl esterase (PME, Pa_4_7580) GH53 endogalactanase (GAL, Pa_1_16660), and PL1, 3 and 4 pectin lyases (PLs). This corresponds with the high pectin content of SBH (Supplementary Table 1). The only exception was GH93 exoarabinanase (ABX, Pa_5_950) being most abundant in WS cultures. *P. anserina* genome harbours one putative GH78 RHA, but this enzyme was not identified from the studied secretomes and the corresponding activity was not detected from the culture liquids. Enzymes targeted for starch cleavage, including GH13 amylases (AMYs),

GH15 GLAs and GH31 α -glucosidases (AGDs), were also most abundant in SBH enzyme mixtures (Fig. 4, Supplementary Table 1). However, no GLA activity was detected from the *P. anserina* culture supernatants, which may be due to the corresponding enzymes not being active on the synthetic *p*NP-linked substrate. Most notable was the high production of GH31 AGDs.

From the large array of putative lignin acting oxidative enzymes encoded by the genome of *P. anserina*, the relatively highest portion of enzymes were detected in CSH cultures (Fig. 4). Three out of 10 putative AA1_3 laccase-like MCOs were identified in the cultures. Of these, Pa_1_15470 showed the highest production especially in both studied time points on SBH. In addition, several H₂O₂-producing enzymes were detected in the cultures, namely 11 AA3_2 glucose-methanol-choline oxidoreductases (GMCs) and an AA5_1 glyoxal oxidase (GLX). They were abundant in the 3-day CSH culture supernatant, in which two of the GMCs and the GLX were among the 15 highest produced plant cell wall acting proteins. Notable was that one laccase and five GMCs were unique for 3-day CSH secretomes (Fig. 3). While H₂O₂ is an essential factor in efficient breakdown of lignocellulose, it is a harmful compound to fungi of which the concentration must be tightly regulated. In accordance with the detection of H₂O₂-producing enzymes, catalase CATB (Pa_7_1610) was detected from all studied *P. anserina* cultures. Interestingly, a putative aromatic peroxygenase (APO, Pa_0_370) was produced in all studied substrates and was among the 15 most abundant plant cell wall degrading enzymes in all samples in the later time point (Supplementary Table 1).

4 Discussion

As a late coloniser of herbivore dung, the ascomycete fungus *P. anserina* is able to use recalcitrant parts of plant cell walls as its carbon source. This feature together with the

exceptionally large repertoire of CAZymes encoded by its genome, makes studying the *P. anserina* plant cell wall degrading enzyme machinery attractive. With the current interest for efficient plant biomass converting enzyme cocktails, the aim of this study was to find culture conditions in which *P. anserina* produces enzyme sets that are able to hydrolyse various plant biomass feedstocks.

Our results show that compared to enzyme mixtures from CSH and WS containing cultures of *P. anserina*, the enzyme cocktail produced on SBH, a major by-product from soybean processing industry [19], was outstanding in its ability to release reduced sugars from CSH, SBH and acid-pre-treated WS. Results obtained with e.g. the enzyme mixtures produced by *Aspergillus niger*, *Penicillium subrubescens* and *Trichoderma reesei* have indicated that saccharification of plant biomass is more effective when the enzyme mixtures are produced on the corresponding feedstocks [20,21]. Also, enzyme supernatants produced by *Trichoderma atroviride* TUB F-1663 mutant on steam-pretreated spruce, WS and sugarcane bagasse yielded comparable amounts of glucose in the hydrolysis of the same feedstocks [22], but none of them was distinguished by superior performance. In contrast to this, the results of our study suggest that SBH, with the most variable chemical composition of the studied substrates, induces a wider array of plant cell wall degrading enzymes of *P. anserina* with robust ability to hydrolyse different plant biomass feedstocks.

Several hydrolytic cellulases from GH1, GH3, GH5, GH6 and GH7 families were present in all cultures showing their importance in the degradation of cellulose. This is in accordance with the results reported from the *P. anserina* secretomes produced in Avicel and sugar beet pulp [23]. Putative secretion signal sequences could not be determined for all the gene models selected for the study, including one BGL that was highly produced in CSH and WS, thus indicating intracellular location for these proteins. However, part of the proteins lacking a secretion signal may also be secreted non-classically as suggested earlier for

Aspergillus nidulans [24]. In addition, several putative cellulose oxidising AA9 LPMOs were present in all *P. anserina* enzyme mixtures used for the saccharification, but in general they were detected in higher amounts in the later time point of SBH than of CSH and WS containing cultures. Also the number of unique LPMOs was highest in SBH supernatants. LPMOs are copper-dependent enzymes that oxidatively cleave glycosidic bonds in various polysaccharides after activation by an electron donor [25–27]. They are ubiquitous in fungi among which the *P. anserina* genome is exceptionally rich with 33 genes encoding putative AA9 LPMOs, thus resembling genomes of plant cell wall degrading basidiomycetes [28]. In a previous study, no peptides corresponding to AA9 LPMOs were detected from the Avicel cultures of *P. anserina* and only seven of these proteins were present in sugar beet pulp cultures [23]. All these proteins were produced in our study.

In line with the high production of LPMOs, all three *P. anserina* putative AA8-AA3_1 CDHs were also detected in the studied cultures. CDHs act as potential electron donors for LPMO oxidative catalysis, and a striking feature of *P. anserina* is that its genome encodes three CDHs, which most often are present as one to two copies in fungal genomes [28,29]. Two of these, Pa_0_280 and a CBM1 containing Pa_7_2650, have been heterologously expressed and biochemically studied [30,31]. The influence of Pa_7_2650 CDH in saccharification of wheat straw has also been investigated as a supplement to industrial *T. reesei* enzyme cocktail [30]. In our study, this CDH was the among the 15 most abundant plant cell wall acting enzymes in all studied conditions highlighting their function in the lignocellulose degradation. Four *P. anserina* LPMOs have been shown to function together with CDH on cellulose regiospecifically and release a different range of oxidised products [31,32]. All of these characterised LPMOs that carry a CBM1 (Pa_1_16300, PaLPMO9E; Pa_2_6530, PaLPMO9A; Pa_4_1020, PaLPMO9H and Pa_7_3160, PaLPMO9B) were produced in all three plant biomass substrates used in the current study. In

addition, three of the characterized *P. anserina* LPMOs have been shown to target different components of plant cell wall, i.e. cellulose, soluble oligosaccharides and hemicellulose [31], thus further supporting their role in the plant biomass degradation by *P. anserina*.

One feature, which distinguished SBH enzyme mixtures from those produced on CSH and WS, was the abundance of the enzymes putatively acting on hemicelluloses. These included one GH5_7 (Pa_6_490) and one GH26 (Pa_5_1950 with a CBM35) mannanases, which were highly produced in SBH. These enzymes (*PaMan5a* and *PaMan26A*, respectively) have been shown to improve the saccharification of WS and spruce softwood when added to the enzyme cocktail of the industrial *Trichoderma reesei* CL847 strain [33]. Especially, *PaMan5a* (Pa_6_490) showed up to 28% improvement of spruce hydrolysis [33]. The same isoenzyme was unique in the SBH enzyme mixture that resulted in superior saccharification in this work, thus supporting the importance of this mannanase in the pretreatment of different lignocellulosic substrates. In addition, crystal structures of these mannanases have been solved, and they have been suggested to act synergistically in hydrolysis of mannan polymers [34], which is supported by the results of our study.

Several xylanolytic enzymes were abundant in SBH cultures, while their production was more variable in CSH and WS cultures. In contrast, low amount of xylan acting enzymes has been reported from the *P. anserina* secretomes from Avicel and sugar beet pulp (Poidevin et al. 2014). In addition, GH2 LACs, and GH43 and GH51 ABFs, which can hydrolyse several polysaccharides, i.e. xylan, xyloglucan and pectin, were scarce in CSH and WS proteomes. However, at the later time point, different GH43 enzymes were detected in the substrates, which highlights the interesting features of that CAZy family, which has recently been reorganized into subfamilies [35]. Two *P. anserina* ABFs (GH51 Pa_5_11670 and GH62 Pa_0_1370) and one XLN (GH11 Pa_2_13730 with a C-terminal CBM1) have been recombinantly produced in *Pichia pastoris* and biochemically characterized [33]. However,

these enzymes were not among the highest representatives from their respective CAZy families in our study.

P. anserina genome contains several genes encoding candidate esterases assigned to families CE1, CE15 and CE16. In addition to that, *P. anserina* genome encodes 14 putative FAEs [36]. In the current work, putative CE1 FAEs and AEs were evenly present in all studied samples and did not exhibit high level production, while two CE15 GEs (Pa_0_910 and Pa_5_11620) were more abundant in the *P. anserina* proteome from 3-day SBH cultivations compared to CSH and WS. GEs are suggested to release xylan that is connected to aromatic alcohols of lignin by ester bonds [37], and recombinant Pa_0_910 has been demonstrated to cleave the ester bond between synthetic lignin alcohols and 4-*O*-methyl-D-glucuronic acid [38]. These glucuronic acid units have been shown to be present in SBH [19]. A representative from family CE16 containing a CBM1 (Pa_5_11290) was also more highly present in proteomes from SBH than from CSH and WS, and interestingly, this enzyme has been shown to possess versatile activities towards polymeric xylan [39].

Although the genome content of *P. anserina* indicates a weak potential for the degradation of pectin, e.g. by absence of GH28 pectin hydrolases and a lower number of pectin lyases compared to *A. niger* [6], the secretome analysis from SBH cultures showed abundant production of different pectin acting enzymes on this pectin rich substrate. In accordance with this result, good growth of *P. anserina* has been shown on soy pectin, although poor growth on other pectins and pectin structural elements was reported [40]. This suggests that the (partial) removal of pectin is essential for efficient degradation of SBH. Also sugar beet pulp, another plant biomass with high pectin content, has been shown to induce high pectinolytic activity and production of pectin acting enzymes in *P. anserina* [23]. The high amount of GH93 ABX produced in WS cultures could be due to the possible activity of this enzyme towards arabino-oligomeric side chains of xylan, although its main

role appears to be the degradation of rhamnogalacturonan I [40]. Enzymes targeted for starch cleavage, including GH13 AMYs, GH15 GLAs and GH31 AGDs, were also most abundant in SBH enzyme mixtures. Most notable was the high production of GH31 α -glucosidases. *P. anserina* has been shown to produce GH31 AGD Pa_2_60 in soluble starch [41], but this enzyme was not detected in our study.

From the laccase-like MCOs of *P. anserina*, Pa_1_15470 was the most highly produced protein especially in both studied time points on SBH. In a laccase gene deletion study, this enzyme was shown to have an effect on the optimal growth of *P. anserina* on *Guibourtia demeusei* wood shavings, thus suggesting a role in lignin depolymerisation or tolerance to lignin-derived compounds (Xia et al. 2014). In a proteomic study of *P. anserina* secretomes, this protein was induced in microcrystalline cellulose (Avicel) containing cultures, but was not detected in sugar beet pulp cultures of *P. anserina* (Poidevin et al. 2014).

Several AA3_2 GMCs and an AA5_1 GLX were produced in the studied *P. anserina* cultures. It has been suggested that, in addition to assisting lignin-modifying peroxidase activity or participating in Fenton-chemistry based lignocellulose degradation, H₂O₂-producing enzymes may participate in the electron transfer mechanism for LPMOs with several target polymers in lignocellulose [42,43]. Accordingly, several putative AA9 LPMOs were present in all *P. anserina* secretomes. Although the enzyme mixtures from CSH contained the highest proportion of putatively lignin oxidising activities, especially GMCs, they did not assist in the saccharification of the different plant biomass feedstocks as CSH supernatant was only able to hydrolyse SBH.

Also, a catalase (Pa_7_1610) was detected from all studied cultures, thus implying participation in the regulation of the concentration of extracellular H₂O₂. In fact, a gene deletion study of *P. anserina* catalases suggested that CATB (Pa_7_1610) is a major

contributor in protecting fungal cells from reactive oxygen species [44]. Interestingly, *P. anserina* showed abundant production of a putative APO especially in the later time point of all studied substrates. *P. anserina* possesses three putative APO encoding genes [6,45], and although the biological relevance of these enzymes is not known [46], it may suggest a role in detoxification of lignocellulose-derived aromatic compounds.

5 Conclusions

Our study shows that SBH as a carbon source triggered *P. anserina* to secrete an enzyme mixture that was superior in saccharification of the tested plant biomasses, i.e. CSH, SBH and acid pre-treated WS, when compared to the enzymes from CSH and WS cultures. Interestingly, SBH was hydrolysed by all enzyme mixtures produced by *P. anserina*. The detailed proteomics analysis showed that the main differences in the enzyme sets were detected in xylan, mannan and pectin acting enzymes, which were relatively abundant in the supernatants originating from SBH cultures. These results support the potential of *P. anserina* as a versatile of source plant cell wall degrading enzyme activities for plant biomass pretreatment for biorefinery applications.

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Supplementary data

Supplementary Figure 1 Extracellular proteins from liquid cultures of *P. anserina*. The proteins from the cultures supplemented with (1) 3% CSH, (2) 3% WS and (3) 3% SBH after 1 to 4 days of growth were separated by SDS-PAGE. M, molecular mass standard.

Supplementary Figure 2 Variable factor map based on the PCA result from 1,487 proteins abundance (approximated with peptide spectra counts, PSMs) across different samples and their duplicates. CSH, cotton seed hulls; WS, wheat straw; SH, soybean hulls.

Supplementary Table 1 Details on the analysis of the proteomics data.

Supplementary Table 2 Monosaccharide composition (mol%) of the plant biomasses used in this work.

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Figure captions

Figure 1 Extracellular CAZyme activities detected from the culture supernatants of *P. anserina* during 4-day cultivation in liquid cultures with A) 3% cotton seed hulls (CSH), B) 3% soybean hulls (SBH) and C) 3% wheat straw (WS) as sole carbon sources. Cellobiohydrolase (CBH), β -glucosidase (BGL), β -1,4-D-galactosidase (LAC), α -arabinofuranosidase (ABF) and α -1,4-D-galactosidase (AGL) activities were determined by using *p*-nitrophenol (*p*NP) linked substrates, and carboxymethyl cellulase (CMCase) and endoxylanase (XLN) activities by measuring the amount of released reducing sugars as glucose equivalents. The vertical bars show the standard deviation of two biological replicate cultures and three technical replicate reactions.

Figure 2 The amount of released reducing sugars from 3% cotton seed hulls (CSH), 3% soybean hulls (SBH) and 3% wheat straw (WS) incubated with the extracellular enzyme mixtures produced by *P. anserina* after 3 days of growth in 3% CSH, 3% SBH and 3% WS supplemented cultures. The vertical bars show the standard deviation of two biological replicate cultures and three technical replicate reactions.

Figure 3 Distribution of the plant cell wall degradation related extracellular enzymes produced by *P. anserina* over the different plant cell wall compounds they putatively act on. The proteins were identified from cotton seed hull (CSH), soybean hull (SBH) and wheat straw (WS) containing cultures after 1 and 3 days of growth. The size of the pie chart reflects the amount of total extracellular protein in the culture liquid. The details are given in Supplementary Table 1E.

Figure 4 Unique and common enzymes related to plant cell wall degradation detected from the different cultures of *P. anserina*. The unique proteins in each sample are listed next to the Venn graph. The font colour of the protein corresponds to the plant cell wall polymer it putatively acts on according to the legend of Fig. 3. Cotton seed hulls, CSH; soybean hulls, SBH; wheat straw, WS. To construct the Venn graphs, all proteins with a lower PSM value than 2 were excluded from the analysis. The details are given in Supplementary Table 1D.

ABF, α -arabinofuranosidase; AE, acetyl esterase; AGD, α -glucosidase; AGL, α -1,4-D-galactosidase; AGU, α -glucuronidase; AMY, amylase; BGL, β -1,4-glucosidase; BXL, β -xylosidase; CBH, cellobiohydrolase; EGL, endoglucanase; FAE, feruloyl esterase; GAL, endogalactanase; GE, glucuronoyl esterase; GMC, glucose-methanol-choline oxidoreductase; GUS, β -glucuronidase; LAC, β -1,4-D-galactosidase; LPMO, lytic polysaccharide monooxygenase; MAN, endo- β -1,4-mannanase; MCO, laccase-like multicopper oxidase; PLY, pectin lyase; PME, pectin methyl esterase;